INTERACTIONS OF *PSEUDOMONAS AUREOFACIENS* PMS382 WITH PEA SEEDLINGS GROWN UNDER STERILE CONDITIONS

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ABSTRACT

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Pseudomonas aureofaciens PMS382, a bacterial strain with strong antifungal activity, was investigated to determine its ability to associate with pea seedlings in a germ-free system. Electron microscopy of pea seedlings inoculated with PMS382 revealed the bacterium associated with the seedling roots. The inoculated plants exhibited reduced growth (fresh shoot weight, root length, shoot length and total dry weight were reduced significantly compared to uninoculated control plants) and necrosis of the cotyledons and roots. Viable counts of plant-associated bacteria revealed survival of the original inoculum and an increase in cfu in many instances. Electron microscopy of plant tissue samples revealed the highest numbers of bacteria were present in the root hair zone. Specific staining revealed the presence of polysaccharide between the root surface and associated PMS382 cells in colonised root zones.

KEYWORDS: *Pseudomonas aureofaciens* - pea - electron microscopy - bacterial attachment.

INTRODUCTION

A strain of *Pseudomonas aureofaciens*, PMS382, with strong inhibitory activity against a range of fungi was originally isolated during screening of bacteria subcultured from mushroom compost (Rainey 1989). Subsequently, PMS382 was shown to produce a number of compounds known to inhibit fungi including phenazines and HCN (Gilpin & Palmer 1992). Four mutants with deficiencies in the production of some or all of these compounds were isolated by transposon mutagenesis (Gilpin & Palmer 1992). The mutations leading to loss of wild-type antifungal activity (Afa$^-$ mutants) are in each mutant due to a single Tn*5* insertion in the PMS382 chromosome (G. Hirst and B. Palmer, unpublished data). The strong antifungal activity of PMS382 (Rainey 1989, Gilpin & Palmer 1992) and the fact that other strains of *P. aureofaciens* have been isolated that have the ability to suppress plant pathogenic fungi (Vincent *et al.* 1991, Thomashow *et al.* 1990) suggest PMS382 may have potential as a biological control agent.

The fluorescent pseudomonads, of which *P. aureofaciens* is a member, have a number of desirable traits for use in the biological control of plant diseases. They are commonly found in the rhizosphere and survive well there, produce antifungal compounds, have an aerobic metabolism and can be subjected to genetic manipulation successfully to allow study of their inhibitory action (Maurhofer *et al.* 1992, Georgakopoulos *et al.* 1994) and their potential for manipulation to enhance or focus their protective ability.

Although other workers have shown that PMS382 cells can adhere to fungal hyphae (Rainey 1989) and plant roots (P. B. Rainey) in this study we aimed to test the interaction of PMS382 with a common crop plant under defined conditions. Here we report experiments performed to study the interaction of PMS382 with pea plant roots in a sterile system and the effects this had on plant growth and bacterial survival. Peas were chosen as a convenient

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test plant because of their significance as a food crop and ease of growth (Hebblethwaite *et al.* 1985). Also peas are infected by the fungus *Aphanomyces euteiches*, a common pea pathogen which grows well under laboratory conditions and has been used in *in vitro* studies of PMS382 (Gilpin & Palmer 1992). The development of this sterile system will allow the future study of the interaction of PMS382 Afa⁻ mutants with plants and fungi. This work provides essential data on inoculum and growth conditions which are required prior to proceeding to trials in non-sterile plant growth media and field experiments.

MATERIALS & METHODS

BACTERIAL AND FUNGAL STRAINS

The strains used in this work, liquid and solid growth media and stock culture storage conditions are described fully by Gilpin and Palmer (1992).

IN VITRO ANTIBIOSIS

The solid growth medium ½PDA/BH (Gilpin & Palmer 1992) was used to determine fungal inhibition *in vitro* as follows. A plug of *A. euteiches* was placed in the centre of a ½PDA/BH plate and incubated at 25°C for 2-3 days by which time the mycelium was clearly growing quickly. The bacterial strains to be tested for ability to inhibit fungus were inoculated in 15 mm long streaks approximately 10 mm from the edge of the mycelium and the edge of the petri dish. *Escherichia coli* DH5α (Woodcock *et al.* 1989) was used as a negative control. The test plates were examined for fungal inhibition at regular intervals.

IN VITRO ANTIFUNGAL ACTIVITY OF PMS382

The stock cultures of *P. aureofaciens* PMS382 had been stored in 15% glycerol, 85 % LB broth at -70°C for at least 2 years prior to this work commencing. Tests of the antifungal activity of PMS382 against *A. euteiches* as performed by Gilpin and Palmer (1992) confirmed their observations and no loss of activity was observed on reviving these cultures (data not shown).

GERMINATION AND GROWTH OF PEA PLANTS

Pea seed of *Pisum sativum* var. William Massey were selected that were no more than half the standard deviation from the mean pea weight (0.26 - 0.30 g) and soaked in 50 ml of 20% bleach plus 100 μl of Triton X-100 (to break the surface tension, ensuring sterilisation of the entire seed surface) for 15 min. The peas were washed 3 times in sterile distilled water (SDW) and then left to soak in the dark at room temperature until the emerging root could be observed (approx. 3 days). An overnight culture of PMS382 was washed then serially diluted in minimal medium without glucose (MM-G, Davis & Mingioli 1950) and the pea seedlings were immersed in a suspension of ~2 x 10^6 cfu ml^{-1}. Half the peas were soaked in this solution for 30 min. The remaining peas were soaked in sterile MM-G as a control.

Inoculated and sterile pea seeds were planted half buried in 3 cm of sterile vermiculite which had been autoclaved 3 times in 30 min cycles over 3 consecutive days in sterilised plastic 150 mm x 65 mm cylindrical tissue culture containers (40 ml of SDW was added to moisten the vermiculite). The plants were grown for 3 weeks in controlled growth rooms at 20°C with 16 hrs light/day (15,000 lux). The seedlings were harvested after 3 weeks and data including dry and fresh weights of the seedlings, root and shoot weight and length were recorded. Bacterial viable counts were performed just after inoculation and following harvesting and some root tissue was set aside for examination by electron microscopy.

PREPARATION OF ROOT TISSUE SAMPLES FOR ELECTRON MICROSCOPY (EM)

i) Critical point drying of specimens for the scanning EM (SEM)

Root tissue sample specimens were fixed by immersion in FAA (an aqueous solution of 3% formaldehyde, 5% acetic acid and 31.5% ethanol). Dehydration of the specimens was achieved in a three stage process. Firstly water was replaced by ethanol by placing the specimens successively in aqueous solutions of 30, 50, 70, 80, 90 and finally 100% ethanol each for 10 mins. Ethanol was then replaced by acetone in the same way as the ethanol was introduced to the specimens. The final step of critical point drying was achieved by placing the specimens in a chamber and pressurising to 600 kPa with CO_2 for 10 mins. The CO_2 was flushed through the chamber for 5 mins. This gas flushing was repeated at 10 min intervals at least 5 times or more, if acetone could still be detected in the escaping gas.

The chamber was then fully sealed and heated to 32°C, using the chamber water jacket and hot water, and the pressure reached approx. 1600 kPa. The temperature and pressure was kept constant for 10 mins (CO_2 was vented if necessary)and then the pressure was released over a period of 10 mins. The dehydrated specimens were removed from the pressure chamber, mounted on an aluminium stub and coated with a 50 nm layer of gold in a sputter coater (SEM Coating Unit E5000, Ploaron Equipment Ltd) and viewed with a Cambridge stereoscan model 250 M2 SEM.

ii) Freeze drying of specimens for the SEM

Specimens were fixed overnight in FAA at room temperature and dehydrated in an ethanol gradient as described above. The specimens were then freeze dried in liquid nitrogen, mounted on an aluminium stub and gold coated before viewing in the SEM as described above.

iii) Preparation of samples for examination in the Transmission EM (TEM)

Plant tissues samples were subjected to primary fixation in 3% glutaraldehyde in 0.075 M phosphate wash buffer (PWB, 21 mM $Na_2HPO_4.2H_2O$, 54 mM $NaH_2PO_4.2H_2O$ pH 7.2) for 2 hours, followed by 3 x 10 min washes in fresh PWB. The samples were then soaked in 1% osmium tetroxide in PWB for 2 hours. Dehydration was achieved by soaking in stepwise 20% increments (20 - 100%) of acetone with 2 further washes in 100% acetone. The samples were infiltrated with 30% Spurr's resin (Spurr 1969) overnight with slow rotation, then immersed in 75% resin for 4 hrs, before embedding in 100% Spurr's resin overnight at 70°C. Blocks of embedded sample were mounted on stubs and thin sections cut using a Spencer "820" microtome and stained in uranyl acetate in 50% ethanol for 10 min and lead citrate for 5 min (Sato & Shamoto 1973) or tested for the presence of polysaccharides using Thiéry's stain (Thiéry 1967).

DATA ANALYSIS

The statistical packages of the Statistical Analysis System (SAS Institute Inc. Cary, North Carolina) General Linear Models were used to test hypotheses and compare data sets.

RESULTS

INTERACTIONS OF PMS382 WITH PEA SEEDLINGS

Eighteen germinating peas were inoculated with PMS382 and allowed to grow as described above for 3 weeks. A further 18 peas were left uninoculated and treated identically as controls. After 3 weeks growth the majority of the seedlings inoculated with PMS382 were stunted or dead, while the control seedlings were healthy and had grown much larger than the inoculated plants. The seedlings were harvested and the parameters of fresh and dry total, shoot, root and seed weight and shoot and root length recorded (Fig. 1). Statistical analysis revealed that fresh shoot weight, shoot length and root length all differed significantly between the control and PMS382 inoculated seedlings. Bacterial counts revealed that a mean of 4.2×10^8 cfu of PMS382 were present per gram fresh weight of root tissue for the inoculated plants after 3 weeks growth compared to 8.1×10^6 cfu g^{-1} at inoculation. Two of the inoculated seedlings had not suffered damage and it was found that these seedlings had a lower number of PMS382 cells, 6.0×10^7 cfu g^{-1} fresh weight of root tissue.

BACTERIAL ATTACHMENT TO THE ROOT SURFACE

In order to examine if PMS382 cells were associated with the root surface of inoculated peas root tissue samples were examined by SEM. Critical point drying fixation left the root tissue intact but removed much of the bacteria associated with the root. Fixation by freeze drying was less disruptive to the attached bacteria but did more damage to the root tissue samples. Bacterial cells were found to be associated with the zone of extension (ZOE) of the roots and in the root hair zone (RHZ) (Fig. 2). In the latter zone there was evidence for degradation of the root tissue which was not present in the ZOE, nor in the RHZ of the control seedlings. In the ZOE the bacterial cells were present in isolated clusters. In or above the root hair zone the bacterial cells are associated with regions of root tissue damage. Tissue samples from leaves were examined for the presence of bacteria, but only extremely isolated single bacteria were observed and it was concluded no significant colonisation of the leaves was achieved by PMS382.

To examine the nature of the assocation between root and bacterial cells root biopsy samples were stained with Thiéry's stain and examined using the

TEM. Figure 3 clearly shows that a fine network of polysaccharide is present between the bacterial cells and the root surface. This polysaccharide was present in some areas where bacterial cells were not associated with the root. It was not possible to deduce whether this polysaccharide was specifically involved in the attachment of PMS382 cells to the root or if these were areas from which bacteria had been dislodged during fixation. It was not possible to determine whether the polysaccharide was produced by the bacterium or the plant but this is worthy of further investigation.

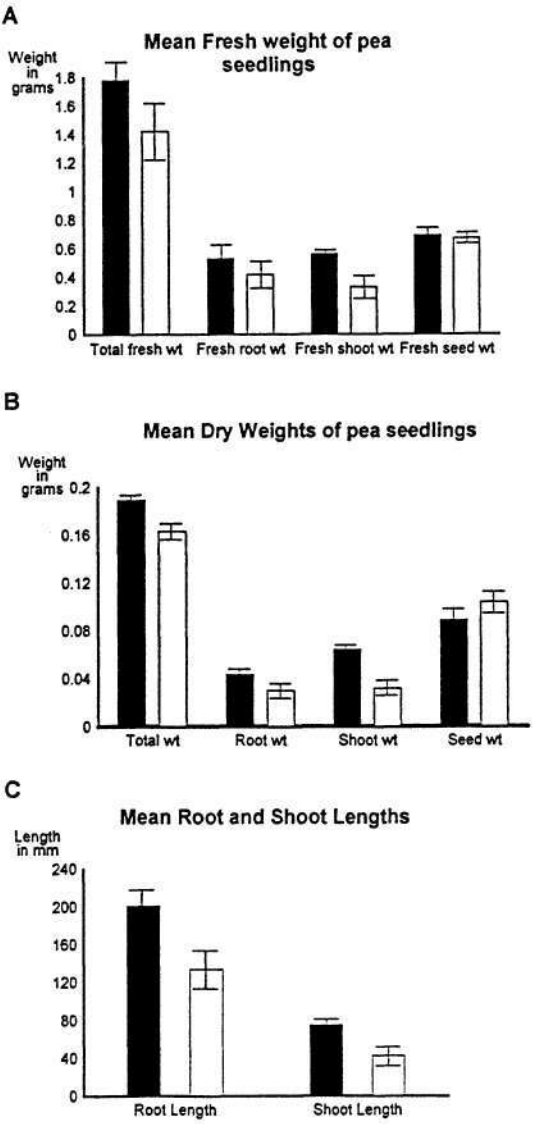

Figure 1. A. Comparison of the fresh weights of pea seedlings grown in sterile conditions and seedlings inoculated with PMS382. B. Comparison of mean the dry weights of pea seedlings grown in sterile conditions and seedlings inoculated with PMS382. C. Comparison of the mean root and shoot lengths of pea seedlings grown in sterile conditions and seedlings inoculated with PMS382. For all graphs ■ = control and □ = PMS382 inoculated.

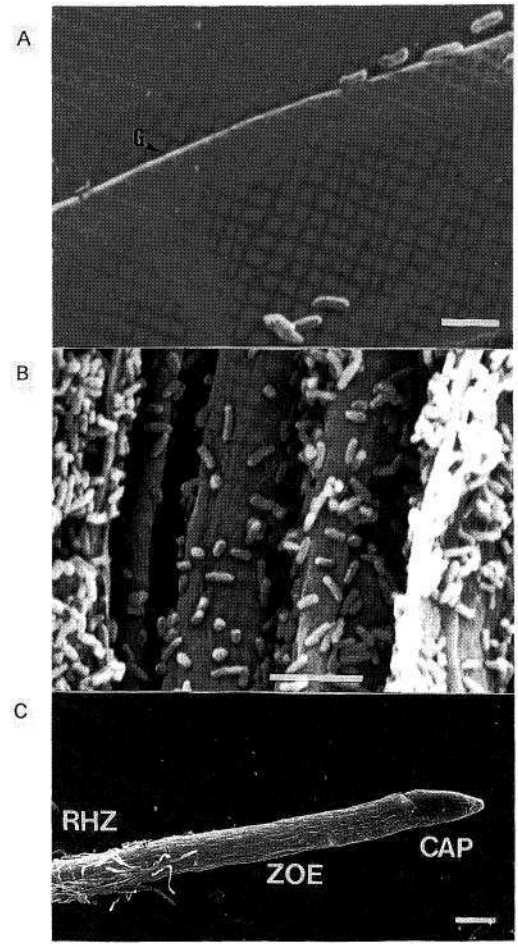

Figure 2. SEM micrographs of pea seedling roots. A. PMS382 associated with the root cells at the zone of extension. G indicates grooves between cells (intercellular spaces). Bar is 2 μm. B. PMS382 cells associated with the root surface below the root hair zone. Bar is 4μm. C. A pea seedling root tip showing the three distinct zones, the root cap on the tip of the root CAP, the zone of extension with distinctive elongated rapidly growing cells ZOE, and the root hair zone RHZ. Bar is 400 μm.

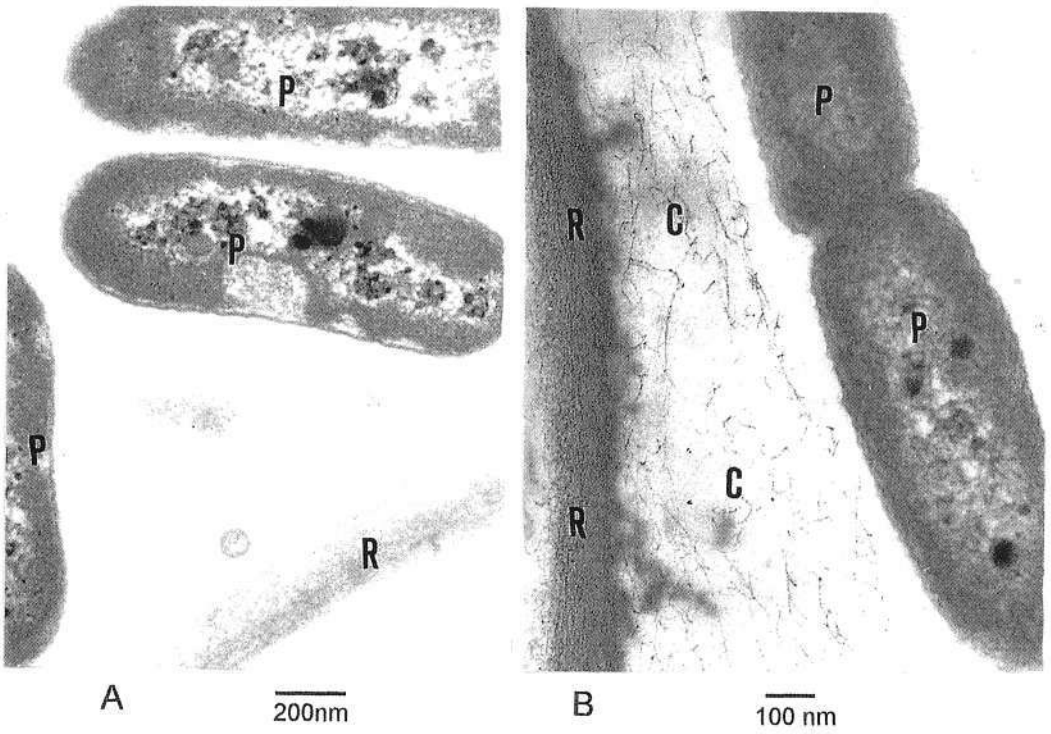

Figure 3. TEM micrographs of pea root sections. A. Lead stained control section showing root cell wall (R) and PMS382 (P) B. Thiéry's stained section showing root cell wall (R), polysaccharide material (C), and PMS382 (P).

DISCUSSION

The development of a sterile system to study the interaction of PMS382 and plants enabled a number of parameters of the association of PMS382 and peas to be determined. It was shown that PMS382 cells can associate with pea roots, but that at the inoculum used here this led to tissue damage and necrosis of the plant. The results obtained suggest that at lower inoculum levels damage to the plant is minimalised. Experimentation to determine if lower inoculum levels of PMS382 alleviates the plant pathogenic effect is continuing. Colonisation density varied along the root depending on the root surface morphology. Above the root hair zone the bacteria appeared to have formed lesions on the surface of the root to which they were strongly adhered. No attempt was made to investigate whether PMS382 provided protection for pea seedlings from infection by the pathogenic fungus *Aphanomyces euteiches*, but this is a logical extension of these experiments. First it is necessary to define conditions under which PMS382 does not adversely affect pea growth. A well-controlled titration of the PMS382 inoculum used in growth experiments, such as those described here, plus the potential of micronutrients such as iron to influence the plant-bacterium interaction should provide the necessary data to proceed to 3 biological component experiments incorporating peas, bacteria and fungi.

The nature of the interaction of PMS382 with the root was investigated using Thiéry's stain which revealed the presence of polysaccharide between the bacteria and root cells. However it was not possible to determine if the polysaccharide was of plant or bacterial origin or if the polysaccharide was strictly required for the adherence of PMS382.

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